

In the Specification

Please replace the current title of the application as amended herein: **PRODUCTION OF ATTENUATED CHIMERIC RESPIRATORY SYNCYTIAL VIRUS VACCINES FROM CLONED NUCLEOTIDE SEQUENCES.**

Please replace paragraphs [0054], [0057], [0058], [0065], [0066], [0068], [0069], [0070], [0071], [0074], and [0075] with the corresponding paragraphs provided below, which have been amended to incorporate sequence identifiers for the relevant figures of the application.

[0054] FIGS. 2 and 3 show the construction of cDNA encoding RSV antigenome RNA, where FIG. 2 shows the structures of the cDNA and the encoded antigenome RNA (not to scale). For the purposes of the present Figures, and in all subsequent Examples hereinbelow, the specific cDNAs and viruses used were of strain A2 of subgroup A RSV. The diagram of the antigenome includes the following features: the 5'-terminal nonviral G triplet contributed by the T7 promoter, the four sequence markers at positions 1099 (which adds one nt to the length), 1139, 5611, and 7559 (numbering referring to the first base of the new restriction site), the ribozyme and tandem T7 terminators, and the single nonviral 3'-phosphorylated U residue contributed to the 3' end by ribozyme cleavage (the site of cleavage is indicated with an arrow). Note that the nonviral 5'-GGG and 3'-U residues are not included in length values given here and thereafter for the antigenome. However, the nucleotide insertion at position 1099 is included, and thus the numbering for cDNA-derived antigenome is one nucleotide greater downstream of this position than for biologically derived antigenome. The 5' to 3' positive-sense sequence of D46 (the genome itself being negative-sense) is depicted in SEQ ID NO: 1, where the nucleotide at position four can be either C or G. Also note that the sequence positions assigned to restriction sites in this Figure and throughout are intended as a descriptive guide and do not alone define all of the nucleotides involved. The length values assigned to restriction fragments here and throughout also are descriptive, since length assignments may vary based on such factors as sticky ends left following digestion. Cloned cDNA segments representing in aggregate the complete

antigenome are also shown. The box illustrates the removal of the BamHI site from the plasmid vector, a modification that facilitated assembly: the naturally occurring BamHI-Sall fragment (the BamHI site is shown in the top line in positive sense, underlined) was replaced with a PCR-generated BglII-Sall fragment (the BglII site is shown in the bottom line, underlined; its 4-nt sticky end, shown in italics, is compatible with that of BamHI). This resulted in a single nt change (middle line, underlined) which was silent at the amino acid level. FIG. 3 shows the sequence markers contained in the cDNA-encoded antigenome RNA, where sequences are positive sense and numbered relative to the first nt of the leader region complement as 1; identities between strains A2 and 18537, representing RSV subgroups A and B, respectively, are indicated with dots; sequences representing restriction sites in the cDNA are underlined; gene-start (GS) and gene-end (GE) transcription signals are boxed; the initiation codon of the N translational open reading frame at position 1141 is italicized, and the restriction sites are shown underneath each sequence. In the top sequence (SEQ ID NO. 22), a single C residue was inserted at position 1099 to create an AflII site in the NS2-N intergenic region, and the AG at positions 1139 and 1140 immediately upstream of the N translational open reading frame were replaced with CC to create a new NcoI site. In the middle sequence (SEQ ID NO. 23), substitution of G and U at positions 5612 and 5616, respectively, created a new StuI site in the G-F intergenic region. In the bottom sequence (SEQ ID NO. 24), a C replacement at position 7560 created a new SphI site in the F-M2 intergenic region.

[0057] FIG. 6 shows construction of D46/1024CAT cDNA encoding an RSV antigenome containing the CAT ORF flanked by RSV transcription signals (not to scale, RSV-specific segments are shown as filled boxes and CAT sequence as an open box). The source of the CAT gene transcription cassette was RSV-CAT minigenome cDNA 6196 (diagram at top). The RSV-CAT minigenome contains the leader region, GS and GE signals, noncoding (NC) RSV gene sequences, and the CAT ORF, with XmaI restriction endonuclease sites preceding the GS signal and following the GE signal (5' and 3' sequences are shown, SEQ ID NOS. 25 and 26, respectively). The nucleotide lengths of these elements are indicated, and the sequences (positive-sense) surrounding the XmaI sites are shown above the diagram. A 8-nucleotide XmaI linker was inserted into StuI site of the parental plasmid

D46 to construct the plasmid D46/1024. D46 is the complete antigenome cDNA and is equivalent to D53; the difference in nomenclature is to denote that these represent two different preparations. The Xma-XmaI fragment of the plasmid 6196 was inserted into the plasmid D46/1024 to construct the plasmid D46/1024CAT. The RNA encoded by the D46 cDNA is shown at the bottom, including the three 5'-terminal nonviral G residues contributed by the T7 promoter and the 3'-terminal phosphorylated U residue contributed by cleavage of the hammerhead ribozyme; the nucleotide lengths given for the antigenome do not include these nonviral nucleotides. The L gene is drawn offset to indicate the gene overlap.

[0058] Fig. 7 is a diagram (not to scale) of the parental wild-type D46 plasmid encoding an RSV antigenome (top), and the D46/6368 derivative in which the SH gene has been deleted (bottom). The RSV genes are shown as open rectangles with the GS and GE transcription signals shown as filled boxes on the upstream and downstream ends, respectively. The T7 phage promoter (left) and hammerhead ribozyme and T7 terminators used to generate the 3' end of the RNA transcript (right) are shown as small open boxes. The *Scal*I and *Pad*I fragment of D46 was replaced with a short synthetic fragment, resulting in D46/6368. The sequence flanking the SH gene in D46, and the sequence of the engineered region in D46/6368, are each shown framed in a box over the respective plasmid map (the top and bottom lines of nucleotides represent sequences 4183-4240 and 4611-4691 of SEQ ID NO: 1, respectively). The sequence of the *Scal*-*Pac*I fragment in D46, and its replacement in D46/6368, are shown in bold and demarcated with arrows facing upward. The M GE, SH GS, SH GE and G GS sites are indicated with overlining. The new M-G intergenic region in D46/6368 is labeled 65 in the diagram at the bottom to indicate its nucleotide length. The positive-sense T7 transcript of the SH-minus D46/6368 construct is illustrated at the bottom; the three 5'-terminal nonviral G residues contributed by the T7 promoter and the 3'-terminal U residue are shown (Collins, et al. Proc. Natl. Acad. Sci. USA 92:11563-11567 (1995), incorporated herein by reference). These nonviral nucleotides are not included in length measurements.

[0065] Fig. 17 depicts the D46 antigenome plasmid which was modified by deletion of the SH gene in such a way as to not insert any heterologous sequence into the recombinant

virus. The sequence flanking the SH gene depicted at the top. The MGE, M-SH intergenic (IG), SH GS, SH GE and SH-G IG sequences are shown. The area which was removed by the deletion is underlined, with the deletion points indicated with upward pointing triangles. The described nucleotide segment consists of sequences 4198-4643 of SEQ ID NO: 1. The antigenome resulting from this deletion is D46/6340.

[0066] Fig. 18 depicts the introduction of tandem translation stop codons into the translational open reading frame (ORF) encoding the NS2 protein. Plasmid D13 contains the left end of the antigenome cDNA, including the T7 promoter (shaded box), the leader region, and the NS1, NS2, N, P, M and SH genes. Only the cDNA insert of D13 is shown. The *AatII-AfIII* fragment containing the T7 promoter and NS1 and NS2 genes was subcloned into a pGem vector, and site-directed mutagenesis was used to modify the NS2 ORF in the region illustrated by the sequence. The wild-type sequence of codons 18 to 26 (SEQ ID NO: 27) is shown (the encoded amino acids are indicated below(SEQ ID NO: 28)), and the three nucleotides above are the three substitutions which were made to introduce two termination codons (ter) and an *XhoI* site (underlined) as a marker. The resulting cDNA and subsequent recovered virus are referred to as NS2-knockout (KO).

[0068] Fig. 20 depicts alteration of gene-end (GE) signals of the NS1 and NS2 genes. The cDNA insert of plasmid D13, representing the left hand end of the antigenome cDNA from the T7 promoter (shaded) to the *PacI* site at position 4623, is shown. The *AatI-AfIII* fragment containing the T7 promoter and the NS1 and NS2 genes was subcloned into a pGem vector. It was modified by site-directed mutagenesis simultaneously at two sites, namely the NS1 and NS2 GE signals were each modified to be identical to that found in nature for the N gene. The sequences of the wild-type NS1 (SEQ ID NO: 29) and NS2 (SEQ ID NO: 30) GE signals are shown (and identified by sequence position relative to the complete antigenome sequence of SEQ ID NO: 1), and the nucleotide substitutions are shown above the line (SEQ ID NOS: 31 and 32, respectively). The dash in the wildtype sequence of the NS2 GE signal indicates that the mutation increased the length of the GE signal by one nucleotide.

[0069] Fig. 21 depicts the deletion of the polynucleotide sequence encoding the NS1 protein. The left hand part of the D13 cDNA is shown at the bottom: D13 contains the left hand part of the antigenome cDNA, from the leader to the end of the SH gene, with the T7 promoter immediately upstream of the leader. The sequence on either side of the deletion point (upward arrow) is shown on top; nucleotide numbering corresponds to that of SEQ ID NO:1. The deletion spans from immediately before the translational start site of the NS1 ORF to immediately before that of the NS2 ORF. Thus, it has the effect of fusing the NS1 GS and upstream noncoding region to the NS2 ORF. This precludes the disruption of any cis-acting sequence elements which might extend into the NS1 gene due to its leader-proximal location.

[0070] Fig. 22 depicts the deletion of the polynucleotide sequence encoding the NS2 mRNA. As described above, the left hand part of the D13 cDNA is shown along with the sequence on either side of the deletion point (upward arrow); nucleotide numbering corresponds to that of SEQ ID NO:1. The deletion spans from immediately downstream of the NS1 gene to immediately downstream of the NS2 gene. Thus, the sequence encoding the NS2 mRNA has been deleted in its entirety, but no other sequence has been disrupted. The resulting cDNA and subsequent recovered recombinant virus are referred to as ΔNS2.

[0071] FIG. 23 depicts the ablation of the translational start site for the secreted form of the G protein. The 298-amino acid G protein is shown as an open rectangle with the signal-anchor sequence filled in. The amino acid sequence for positions 45 to 53 (SEQ ID NO: 33) is shown overhead to illustrate two nucleotide substitutions (SEQ ID NO: 34) which change amino acid 48 from methionine to isoleucine and amino acid 49 from isoleucine to valine. The former mutation eliminates the translational start site for the secreted form. The two mutations also create an MfeI site, which provides a convenient method for detecting the mutation. The resulting cDNA and subsequent recovered virus are referred to as M48I (methionine-48 to isoleucine-48).

[0074] FIGS. 25B and 25C illustrate the sequence (positive-sense) in the chimeric rAB virus, namely recombinant RSV strain A2 in which the F and G glycoprotein genes were replaced with those of strain B1. The sequence shown contains part of the SH-G (Part B, SEQ

[ID NO: 35] and F-M2 (Part C, SEQ ID NO: 36) junction. Sequence derived from the strain A2 backbone is shown in lower case, and that from the strain B1 donor is in upper case. The last A2-specific nucleotide at the junction between the A2 and B1 sequence is numbered according to the unmodified recombinant A2 genomic sequence. The SH gene-end (GE) and F GE signals are boxed. The PacI and SphI recognition sites are italicized. IG: intergenic region.

[0075] FIG. 26 illustrates modification the cDNA of the strain B1 G and F genes in order to improve stability during growth in *E. coli*. Two positive-sense sequences are shown: the upper one (labeled "new") is the modified B1 sequence (SEQ ID NO: 37), and the lower ("wt") is the wild-type B1 sequence (nucleotides 5554-5663 of SEQ ID NO: 2). The sequence shown includes the downstream end of the G translational open reading frame (ORF), its encoded amino acids (shown as the single letter code below the sequence, SEQ ID NO: 38), the G GE signal (boxed), the G-F intergenic region, and the F gene-start (GS) signal (boxed). Underlined positions in the new sequence represent substitutions; dashes in the new sequence represent deletions; dashes in the wt sequence indicate an insertion in the new sequence. An MfcI site created in the new sequence is in bold italics. A 47-nucleotide sequence from the G-F intergenic region which was deleted in creating the new sequence is indicated.